

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
12 April 2001 (12.04.2001)

PCT

(10) International Publication Number  
**WO 01/25266 A1**

- (51) International Patent Classification<sup>7</sup>: **C07K 7/08, A61K 38/10, A61P 35/00**
- (21) International Application Number: **PCT/US00/21998**
- (22) International Filing Date: **4 October 2000 (04.10.2000)**
- (25) Filing Language: **English**
- (26) Publication Language: **English**
- (30) Priority Data:  
**60/157,950      6 October 1999 (06.10.1999)      US**
- (71) Applicant (*for all designated States except US*): **PHARMACIA CORPORATION [US/US];** Corporate Patent Department, P.O. Box 5110, Chicago, IL 60680-5110 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (*for US only*): **SHAILUBHAI, Kunwar [US/US];** 15438 Harrisberg Court, Chesterfield, MO 63017 (US). **CURRIE, Mark, G. [US/US];** 404 Mason Ridge Drive, St. Charles, MO 63304 (US).
- (74) Agents: **BENNETT, Dennis, A. et al.;** Pharmacia Corporation, Corporate Patent Department, P.O. Box 5110, Chicago, IL 60680-5110 (US).
- (81) Designated States (*national*): **AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.**
- (84) Designated States (*regional*): **ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).**
- Published:**  
— *With international search report.*  
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 01/25266 A1

**BEST AVAILABLE COPY**

(54) Title: **UROGUANYLIN AS AN INTESTINAL CANCER INHIBITING AGENT**

(57) Abstract: Disclosed is a method of retarding the development of polyps and prevention, inhibition and treatment of cancer in the intestine of a subject by administration of a composition comprising a peptide with the active domain of uroguanylin, or any agonist peptide or compound binding to the guanylate cyclase receptor GC-C in the intestine.

## UROGUANYLIN AS AN INTESTINAL CANCER INHIBITING AGENT

This application claims priority from U.S. provisional application # 60/157,950, filed October 6, 1999, which is incorporated herein by reference.

5

BACKGROUND OF THE INVENTION

The present invention relates to the use of certain peptides, more particularly the use of uroguanylin, prouroguanylin, guanylin, and other like peptides to retard the development of polyps and prevent, inhibit or treat  
10 cancer in the intestine.

The pathogenesis of colorectal cancer is characterized as a multistep process that begins with increased proliferation and/or decreased apoptosis of colorectal epithelial cells resulting in generation of polyps, followed  
15 by adenoma formation and ultimately to adenocarcinoma. Certain individuals develop multiple colorectal adenomas and subsequent carcinomas early in life because of a genetic defect in the APC gene responsible for causing a condition called familial adenomatous polyposis (FAP). Dihlmann et  
20 al, *Dominant negative effect of the APC 1309 mutation: a possible explanation for genotype-phenotype correlations in familial adenomatous polyposis*, Cancer Res. 1999 Apr. 15, 59(8): 1857-60. Chemoprevention has evolved during the last decade as a viable strategy for cancer prevention, with the  
25 aim of controlling the development of cancer through pharmacological and/or dietary intervention prior to the appearance of a clinically detectable tumor. Reddy, B.S. (1997) *Chemoprevention of colon cancer by dietary administration of naturally-occurring and related synthetic*  
30 *agents*, Adv. Exp. Med. Biol. 400B:931-936.

Uroguanylin and guanylin are structurally related enteric peptide hormones that are secreted intraluminally by different types of cells, include enterochromaffin, goblet and others within the intestinal mucosal lining. A receptor  
35 for theses peptides that has been identified at the molecular level is a transmembrane form of guanylate cyclase

(GC) known as GC-C. Krause, W.J. et al, *The guanylin and uroguanylin peptide hormones and their receptors*, Acta. Anat. (Basel) 160:213-231 (1997). GC-C receptors are localized on the luminal surface of enterocytes throughout the GI tract. Swenson, E.S. et al, *The guanylin/STa receptor is expressed in crypts and apical epithelium throughout the mouse intestine*, Biochem. Biophys. Res. Commun. 225:1009-1014 (1996). Binding of uroguanylin or guanylin to the extracellular domain of GC-C receptors stimulates intracellular production of the second messenger cGMP, resulting in activation of cystic fibrosis transmembrane conductance regulator (CFTR), the apical membrane channel for efflux of chloride from enterocytes lining the intestinal tract. Forte, L.R. et al, *Salt and water homeostasis: uroguanylin is a circulating peptide hormone with natriuretic activity*, Am. J. Kidney Dis. 28:296-304 (1996). Activation of CFTR chloride channel proteins and the subsequent enhancement of transepithelial secretion of chloride leads to stimulation of sodium (Na<sup>+</sup>) and water secretion into the intestinal lumen. Forte, L.R. et al, *Guanylin regulatory peptides: structures, biological activities mediated by cyclic GMP and pathobiology*, Regul. Pept. 81:25-39 (1999). Therefore, one of the major physiological functions of these hormones is the regulation of fluid and electrolyte transport in the gastrointestinal (GI) tract by serving as paracrine regulators of CFTR activity.

The precursor of uroguanylin is prouroguanylin, which is broken down by endogenous proteases in the intestinal tract to produce the active uroguanylin. Chymotrypsin activates prouroguanylin to cleave it into its active form of uroguanylin. Forte, et al, *Salt and Water Homeostasis: Uroguanylin Is a Circulating Peptide Hormone With Natriuretic Activity*, Am. J. Kid. Dis. 1996, 28, No.2, '296-304. Uroguanylin is an acid-stable and proteolysis-resistant peptide, which will remain intact to act on the intestinal lumen directly rather than being absorbed

systemically. Uroguanylin and guanylin are produced throughout the intestinal mucosa and in the myocardium. Forte et al, *Salt and water homeostasis: uroguanylin is a circulating peptide hormone with natriuretic activity* Am. J. Kidney Dis. 28:296-304 (1996). Human uroguanylin has been isolated from human urine and has been chemically synthesized by solid phase peptide synthesis as described in U.S. Patent Number 5,489,670 for *Human Uroguanylin*. Additionally, human guanylin has been isolated from human intestinal cells and has been chemically synthesized by solid phase peptide synthesis as described in U.S. Patent Number 5,969,097 for *Human Guanylin*.

Binding of uroguanylin or guanylin to the guanylin cyclase receptor stimulates the intracellular production of the cGMP ultimately resulting in the stimulation of salt and water secretion into the intestinal lumen. Uroguanylin and guanylin receptors are found on the luminal surface of epithelial cells lining the intestinal tract and renal proximal tubules as well as in other organs. Forte et al, *Salt and Water Homeostasis: Uroguanylin Is a Circulating Peptide Hormone with Natriuretic Activity*, Am. J. Kid. Dis. 1996, 28, No. 2, 296-304. Uroguanylin has been found to stimulate increases in cyclic GMP levels in a manner similar to another family of heat stable enterotoxins (STs) secreted by pathogenic strains of E. coli and other enteric bacteria that activate intestinal guanylate cyclase and cause secretory diarrhea, which is a major cause of traveler's diarrhea and many deaths in developing countries. Forte et al, *Lymphoguanylin: Cloning and Characterization of a Unique Member of the Guanylin Peptide Family*, Endocrinology Vol. 140, No. 4, p.1800-1806. These ST peptides act as molecular mimics of the endogenous mammalian peptides of uroguanylin and prouroguanylin. Forte et al, Endocrinology Vol. 140, No. 4, p.1800. Unlike uroguanylin the STs from enteric bacteria do not have a decrease in potency when the pH changes in the colon. STs are more potent than either uroguanylin or guanylin under both acidic and alkaline

conditions. Forte et al, *Guanylin: a peptide regulator of epithelial transport*, The FASEB Journal, vol. 9, 643-650 (1995). Uroguanylin is believed to regulate fluid and electrolyte transport in a manner similar to guanylin and the STs in the GI tract. Therefore, as mentioned in previous publications the human uroguanylin may act as a laxative and be useful in patient suffering from constipation.

10

#### SUMMARY OF THE INVENTION

Among the objects and features of the present invention may be noted the provision of a method of modulating polyps in the intestine of a subject, in need thereof; said "modulating" or "modulation" includes retarding the development of polyps, preventing, treating, and inhibiting polyps. Also, the present invention is directed to a method of preventing, inhibiting and treating cancer in the intestine (small intestine and colon) of a subject in need thereof.

20

Briefly, therefore, the present invention is directed to a process for modulating polyps in the intestine of a subject, in need thereof, which comprises the administration of a peptide including the amino acid sequence:

$X_8$ -Asp- Asp- Cys-  $X_1$ -  $X_2$ - Cys-  $X_3$ - Asn-  $X_4$ -  $X_5$ - Cys-  $X_6$ -  $X_7$ - Cys- $X_9$ ,

25

wherein each of  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$ ,  $X_6$ , and  $X_7$  is an amino acid residue,  $X_8$  and  $X_9$  are independently hydrogen or at least one amino acid residue, and the polypeptide is cross-linked by a disulfide bond between the cystine residue immediately adjacent the amine group of  $X_1$  and the cystine residue immediately adjacent the amine group of  $X_6$  and by a disulfide bond between the cystine residue immediately adjacent the amine group of  $X_3$  and the cystine residue immediately adjacent the carboxy group of  $X_7$ , together with a pharmaceutically acceptable carrier.

30

The invention is further directed to a method for modulation of polyps in a subject, and to a process for the prevention, inhibition or treatment of cancer in the intestinal tract by administration of a pharmaceutical composition comprising any one of or combination of the following peptides: uroguanylin, human uroguanylin, pro-uroguanylin, and human pro-uroguanylin, guanylin, lymphoguanylin, prolymphoguanylin and heat stable enterotoxin, together with a pharmaceutically acceptable carrier.

Additionally, the invention is directed to a process for modulating polyps in the intestine of a subject, and a process for the prevention, inhibition or treatment of cancer in the intestine of a subject, in need thereof, by administration of a pharmaceutical composition comprising any one of or a combination of agonist peptides and/or other agonist compounds to the guanylate cyclase receptor GC-C, together with a pharmaceutically acceptable carrier.

Other objects of this invention will be in part apparent and, in part, pointed out hereinafter.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1(a) depicts the effect of human uroguanylin on the stimulation of  $I_{sc}$  where fresh mouse duodenum consisting of mucosa and submucosa ( $\sim 1\text{cm}^2$ ) was mounted between two halves of Ussing Chambers and bathed on both sides as described. At the arrows, indicated concentrations of TTX, uroguanylin (uroG) and carbachol were added to the apical reservoir. Electrical measurements were monitored with an automatic voltage clamp.

Figure 1(b) depicts the effect of human uroguanylin on the stimulation of  $I_{sc}$  where human intestinal mucosa ( $\sim 1\text{cm}^2$ ) was mounted between two halves of Ussing Chambers and bathed on both sides as described. At the arrows, indicated concentrations of TTX, uroguanylin (uroG) and carbachol were added to the apical reservoir. Electrical measurements were monitored with an automatic voltage clamp.

Figure 2 depicts a graphic demonstration of the effect of human uroguanylin on the inhibition of proliferation of T-84 human carcinoma cells. Cells were inoculated in 96-well plates. After an incubation of 72 hours, indicated concentrations of human uroguanylin were added in the media and cells were allowed to grow until they formed semiconfluent monolayers. Subsequently, 5-bromo-2-deoxyuridine (BrdU) was added (final concentration 100 $\mu$ M) and cells were re-incubated for an additional 24 hours. The incorporation of BrdU was measured at 450 nm as per manufacturer's instructions.

Figure 3 depicts the fragmentation of DNA in T-84, human colon carcinoma cells, after treatment with human uroguanylin as analyzed by electrophoresis using 1.8% agarose gel followed by staining with ethidium bromide. Approximately  $2 \times 10^5$  cells were inoculated in 35 mm dishes and cultured for 7 days. Semiconfluent monolayers were washed with serum-free DMEM, and further incubated with the same media containing indicated concentrations of human uroguanylin. Subsequently, the cells were quickly collected by trypsinization and washed twice with PBS. Harvested cells were immediately used for DNA isolation as per the instructions of the DNA fragmentation analysis kit (Boehringer Mannheim Corp., Indianapolis, IN). The fragmentation of DNA was analyzed by electrophoresis using 1.8% agarose gel followed by staining with ethidium bromide. Apoptotic DNA provided with the test kit was used as positive control, M (lane 1) and a functionally inactive variant of human uroguanylin (V) was used as negative control (lane 6). Different concentrations of uroguanylin, as indicated were examined (lanes 2 to 5).

Figure 4 depicts microscopic slides with semi-confluent monolayers of Caco-2 cells demonstrating the effects of human uroguanylin on the induction of apoptosis. Cells were cultured on microscopic slides until they formed semi-confluent monolayers. Subsequently the cells on slide B were treated with human uroguanylin (1  $\mu$ M) for 48 hours.

Induction of apoptosis was detected by fluorescence microscopy directly after the TUNEL reaction as per the instructions of "In situ cell death detection kit"

(Boehringer Mannheim Corp., Indianapolis, Indiana). Slide A depicts vehicle-treated cells. Slide B depicts uroguanylin-treated cells.

Figure 5(a) depicts a Northern blot analysis demonstrating that the expression of uroguanylin and guanylin is suppressed in human colon carcinoma cells.

Figure 5(b) depicts an RT-PCR followed by Southern blotting demonstrating that the expression of uroguanylin and guanylin is suppressed in human colon carcinoma cells.

Figure 6(a) depicts a graphic demonstration of the enhancement of daily food consumption by Min-mice after oral administration of human uroguanylin. Total food consumption per day (24 hours) by five (5) animals in one cage was determined and used for calculation of total food consumption per mouse per day. Results are expressed as an average  $\pm$  standard deviation.

Figure 6(b) depicts a graphic demonstration of the enhancement of body weight gain by Min-mice after oral administration of human uroguanylin. Body weights of all animals were measured weekly throughout the study. Results are expressed as average  $\pm$  standard deviation of gain in body weight per mouse during the study.

Figure 7 depicts the primary structure of human uroguanylin (*h UroG*) [identified as SEQ. ID. 2], human guanylin (*h Gua*) [identified as SEQ. ID. 3], and bacterial enterotoxins (*E.coli* [identified as SEQ. ID. 4] & *V.cholerae* [identified as SEQ. ID. 5]). Bold and italic letters represent the similar residues in these peptides. These residues are believed to be required for the functional activity of these peptides. *E. coli* ST has three additional residues (Asn-Ser-Ser) and *V.cholerae* has two additional residues (Leu and Ile) at their N-termini. These N-terminal residues make bacterial ST insensitive towards intestinal pH. Two underlined (Asp-Asp) residues are



believed to be important for regulating the functional activity of uroguanylin only at the acidic environment of the intestinal mucosa.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

5 Uroguanylin is secreted naturally by the goblet cells of the intestinal mucosal lining as prouroguanylin, a functionally inactive form, which is then converted to the functionally active uroguanylin in the intestine by endogenous proteases. Uroguanylin is an acid-stable, 10 proteolysis-resistant peptide. Therefore, orally delivered prouroguanylin and uroguanylin will act on the luminal intestinal surface and not be absorbed systemically. Oral administration of uroguanylin, prouroguanylin and other like peptides, containing the amino acid sequences similar to the 15 active domain, are expected to induce apoptosis, cell death, in the intestinal mucosal cell lining. The induced apoptosis in the intestinal mucosal cell lining is expected to retard the incidence of polyp formation and subsequent intestinal cancer. Without intending to be bound by any 20 theory, applicants believe that the peptides of the invention exert their effects by increasing the rate of apoptosis, cell death, in the intestinal mucosal cell lining promoting the perfect balance between the cell proliferation and the programmed cell death thereby retarding the growth 25 of polyps and preventing, inhibiting, and treating cancer in the intestine and other epithelial-derived cancer possessing receptors for guanylin, uroguanylin, lymphoguanylin and STa family of peptides.

The rate of cell proliferation and cell death in the 30 intestinal mucosa is very rapid. The cells of the intestinal mucosa are in a steady state of turnover to insure a perfect balance between cell proliferation and cell death. The constant rapid renewal of the GI tract epithelium fulfills the functions of maintaining the 35 integrity of normal mucosa, repairing and replenishing differentiated epithelial cells that have specialized

functions. The prevention of apoptosis in the intestinal mucosal cells creating an imbalance in the renewal process results in an increased incidence of polyp formation and subsequent intestinal cancer. See Eastwood et al, A review  
5 of gastrointestinal epithelial renewal and its relevance to the development of adenocarcinomas of the gastrointestinal tract, J. Clin. Gastroenterol. 21: S1-11 (1995). The process of apoptosis is known to be suppressed in colon cancer tissues. Baretton, et al, Apoptosis and  
10 immunohistochemical bcl-2 expression in colorectal adenomas and carcinomas. Aspects of Carcinogenesis and prognostic significance, Cancer 77:255-264 (1996).

A major cellular characteristic of the apoptotic process is a marked loss of cell volume, which is directly  
15 related to the movement of ions, with homeostasis being achieved by the balance of osmotic pressure across the plasma membrane. Hoffman, E.K. et al, Membrane mechanisms in intracellular signalling in cell volume regulation, Int. Rev. Cytol. 161:173-262 (1995). Most mammalian cells  
20 achieve and maintain this osmotic pressure through the continuous action of Na<sup>+</sup>/K<sup>+</sup> ATPase pump, which creates a gradient of these monovalent cations across the membrane. Several sources of evidence have implicated a potential role of K<sup>+</sup> efflux in the induction of apoptosis. Hughes, F.M. et  
25 al, Intracellular K<sup>+</sup> suppresses the activation of apoptosis in lymphocytes, J.Biol.Chem. 272:30567-30576 (1997); Hughes, F.M. et al, Potassium is a critical regulator of apoptotic enzymes in vitro and in vivo, Adv. Enzyme Regul. 39:157-171 (1999). First, a bacterial pore-forming cytolysin,  
30 staphylococcal  $\alpha$ -toxin, which selectively permeabilizes plasma membranes for monovalent cations, was found to induce apoptosis. Bhakdi, S. et al, Release of interleukin-1 beta associated with potent cytotoxic action of staphylococcal alpha-toxin on human monocytes, Infect.  
35 Immun. 57:3512-3519 (1989). Second, apoptotic and shrunken cells have been shown to contain much lower levels of intracellular K<sup>+</sup> as compared to that in normal cells.

Hughes, F.M et al, *Intracellular K<sup>+</sup> suppresses the activation of apoptosis in lymphocytes*, J.Biol.Chem. 272:30567-30576 (1997). Third, an intracellular K<sup>+</sup> concentration more than 150mM has been shown to selectively inhibit Caspase-3, a proteolytic enzyme involved in the induction of apoptosis. Hughes, F.M. et al, *Potassium is a critical regulator of apoptotic enzymes in vitro and in vivo*, Adv.Enzyme Regul. 39:157-171 (1999). Finally, suppressing K<sup>+</sup> efflux in whole cells prevents the activation of pro-apoptosis nucleases, whereas enhancing the efflux of this ion facilitates enzymatic activities of these nucleases. Hughes, F.M. 39: 157-171 (1999). Thus, intracellular levels of potassium balance appear to be the critical regulator of apoptosis.

Without intending to be bound by any theory, applicants believe that there is a relationship between K<sup>+</sup> channel activity and uroguanylin-induced apoptosis in colon carcinoma cells. Uroguanylin and guanylin have been shown to stimulate Cl<sup>-</sup> and K<sup>+</sup> efflux to regulate electrolyte and water transport in the GI tract. Recently, heat-stable enterotoxin (STa) of *Escherichia coli*, a GC-C agonist peptide that also increases intracellular accumulation of cGMP and stimulates fluid secretion in the lumen of the intestine, has been shown to increase K<sup>+</sup> efflux and Ca<sup>2+</sup> influx. Bhattacharya, J. et al, *Rise of intracellular free calcium levels with activation of inositol triphosphate in a human colonic carcinoma cell line (COLO 205) by heat-stable enterotoxin of Escherichia coli*, Biochem. Biophys. Acta. 1403:1-4 (1998). Atrial natriuretic peptide (ANP), a peptide that stimulates intracellular accumulation of cGMP by binding to a specific GC receptor, has also been shown to activate K<sup>+</sup> conductance in rat mesangial cells, and to induce apoptosis in cardiac myocytes by a cGMP-dependent mechanism. Cermak, R. et al, *Natriuretic peptides increase a K<sup>+</sup> conductance in rat mesangial cells*, Pflugers Arch. 43:571-577 (1996). Furthermore, pretreatment of rat endothelial cells with either ANP (10<sup>-7</sup>M) or 8-bromo-cGMP(10<sup>-3</sup>M) caused a

marked accumulation of the nuclear phosphoprotein, p53, a tumor suppresser protein known to induce apoptosis in many cell types. Suenobu, N. et al, *Natriuretic peptides and nitric oxide induce endothelial apoptosis via a cGMP-dependent mechanism*, Arterioscler. Thromb. Vasc. Biol. 19:140-146 (1999). Also, CFTR expression is associated with K<sup>+</sup> and Cl<sup>-</sup> efflux and shrinkage of cells, characteristic biochemical changes found in apoptotic cells. Rotoli, B.M. et al, *CFTR expression in C127 cells is associated with enhanced cell shrinkage and ATP extrusion in Cl(-)- free medium*, Biochem. Biophys. Res. Commun. 227:755-61 (1996). Applicants believe that uroguanylin, prouroguanylin, guanylin and other like peptides may induce apoptosis of epithelial cells lining the GI tract mucosa via maintenance of intracellular concentration of K<sup>+</sup> ions as a result of binding to the GC-C receptors. Applicants believe that the binding of the GC-C receptors stimulates the production of cGMP thereby activating the CFTR chlorine channel which causes an increase in K<sup>+</sup> efflux. Thus, the induction of apoptosis is also expected from the administration of agonist peptides which bind to the GC-C receptors, and to other receptors for guanylin, uroguanylin and lymphoguanylin in the intestine.

Additionally, guanylin has been shown to be completely diminished in colon cancer cells and evenly expressed in normal intestinal mucosal cells. This finding suggest that guanylin is involved in the maintenance of colonic differentiation or functions as a tumor modifier gene. Mitchell et al., *Guanylin mRNA Expression in Human Intestine and Colorectal Adenocarcinoma*, Lab. Invest. 1998, Vol. 78, No. 1, 101-108. Recent data demonstrates that the guanylin cyclase receptor known as GC-C receptor is expressed in all primary and metastatic colorectal cancers and it may serve as a specific marker for these tumors. Carrithers, S.L. et al, *Guanylin cyclase C is a selective marker for metastatic colorectal tumors in human extraintestinal tissues*, Proc. Natl. Acad. Sci. USA. 93:14827-14832. By contrast, the

expression of guanylin has been shown to be down-regulated in colorectal cancer tissues and cell lines. Cohen, M.B. et al, *Guanylin mRNA expression in human intestine and colorectal adenocarcinoma*, Lab. Invest. 78:101-108.

5        A study described in the examples to this application shows that uroguanylin is similarly completely diminished in colon cancer cells and evenly distributed in normal intestinal mucosal cells. Additionally, the expression of uroguanylin in human colon cancer and the adjacent normal  
10       tissues has been examined. Thus, the expression of both uroguanylin and guanylin is completely diminished in all human colon cancer specimens examined. This study suggests that either the reduced expression of uroguanylin and/or guanylin leads to or is a result of adenocarcinoma  
15       formation. The applicants also demonstrate that treatment with uroguanylin results in the induction of apoptosis in T-84, human colon carcinoma cells, and that the oral administration of human uroguanylin leads to inhibition in polyp formation in the intestinal tract of Min-mouse, an  
20       animal model for human Familial Adenomatous Polyposis (FAP).

Both guanylin and uroguanylin genes have recently been mapped on the mouse chromosome 4 and to a syntenic position on human chromosome 1p34-35. Sciaky, D. et al, *Mapping of guanylin to murine chromosome 4 and human chromosome 1p34-35*, Genomics 26:427-429 (1995); Whitaker, T.L. et al, *The uroguanylin gene (Guca 1b) is linked to guanylin (Guca 2) on mouse chromosome 4*, Genomics 45:348-354 (1997). This region is frequently associated with the loss of heterozygosity in human colon carcinoma. Leister, I. et al, *Human colorectal cancer: high frequency of deletions at chromosome 1p35*, Cancer Res. 50:7232-7235 (1990). In the min-mouse tumor model, adenoma multiplicity and growth rate are regulated by APC, the tumor suppressor gene, which is also localized to mouse chromosome 4 in a region syntenic with human  
30       chromosome 1p34-36. Dietrich, W.F. et al, *Genetic identification of Mom-1, a major modifier locus affecting Min-induced intestinal neoplasia in the mouse*, Cell 75:631-  
35

639 (1992). The APC gene is mutated in the vast majority of humans with colorectal cancer. Miyoshi, Y. et al, *Somatic mutations of the APC gene in colorectal tumors: mutation cluster region in the APC gene*, Hum. Mol. Genet. 1:229-233 (1992). The principal function of this gene is to regulate cell cycle via the wnt signal transduction cascade. Cadigan, K.M. et al, *Wnt signaling: a common theme in animal development*, Genes Dev. 11:3286-3305 (1997). Thus, the uroguanylin and guanylin peptides may be involved early in the process of colon carcinogenesis.

In accordance with the process of the present invention, therefore, a polypeptide which contains the active domain of human uroguanylin or which binds to the guanylate cyclase receptor GC-C in the intestine of the subject is administered to a subject. While the polypeptide may be administered prophylactically, it will typically be administered to a subject who has been determined to have intestinal cancer, intestinal polyps, or a genetic predisposition for the growth of polyps in the intestine.

In a preferred embodiment of the present invention, the polypeptide is a polypeptide having the sequence as identified in SEQ. ID. 1:

$X_8$ -Asp -Asp -Cys - $X_1$  - $X_2$  -Cys - $X_3$  -Asn - $X_4$  - $X_5$  -Cys - $X_6$  - $X_7$  -Cys- $X_9$ ,

wherein each of  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$ ,  $X_6$ , and  $X_7$  is an amino acid residue,  $X_8$  and  $X_9$  are independently hydrogen or at least one amino acid residue, and the polypeptide is cross-linked by a disulfide bond between the cystine residue immediately adjacent the amine group of  $X_1$  and the cystine residue immediately adjacent the amine group of  $X_6$  and by a disulfide bond between the cystine residue immediately adjacent the amine group of  $X_3$  and the cystine residue immediately adjacent the carboxy group of  $X_7$ . Preferably, the polypeptide is guanylan, uroguanylin, pro-uroguanylin, or another polypeptide which contains the active domain of uroguanylin.

As is known in the art, certain amino acids in a peptide or protein can be substituted for other amino acids having a similar hydropathic index or score and produce a resultant peptide or protein having similar biological activity, i.e., which still retains biological functionality. In making such changes, it is preferable that amino acids having hydropathic indices within  $\pm 2$  are substituted for one another. More preferred substitutions are those wherein the amino acids have hydropathic indices within  $\pm 1$ . Most preferred substitutions are those wherein the amino acids have hydropathic indices within  $\pm 0.5$ .

Like amino acids can also be substituted on the basis of hydrophilicity. U.S. Patent No. 4,554,101 discloses that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein. The following hydrophilicity values have been assigned to amino acids: arginine/lysine (+3.0); aspartate/glutamate (+3.0  $\pm 1$ ); serine (+0.3); asparagine/glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5  $\pm 1$ ); alanine/histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine/isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); and tryptophan (-3.4). Thus, one amino acid in a peptide, polypeptide, or protein can be substituted by another amino acid having a similar hydrophilicity score and still produce a resultant protein having similar biological activity, i.e., still retaining correct biological function. In making such changes, amino acids having hydropathic indices within  $\pm 2$  are preferably substituted for one another, those within  $\pm 1$  are more preferred, and those within  $\pm 0.5$  are most preferred.

As outlined above, amino acid substitutions in the peptides of the present invention can be based on the relative similarity of the amino acid side-chain substituents in the non-active domain of the peptide to create a protein with the same biological activity as the human uroguanylin peptide. Thus,  $X_1$  may be selected from the

group of all amino acid residues, but preferably is selected from the group of amino acid residues consisting of aspartic acid, glutamic acid, glycine, lysine, asparagine, proline, glutamine, arginine, serine and threonine. The more  
5 preferred amino acid residues that may be substituted for  $X_1$  are glutamic acid, aspartic acid, arginine, and lysine. The most preferred amino acid residue that may be used for  $X_1$  is glutamic acid.  $X_2$  may be selected from all amino acid residues, however the preferred amino acid residues for  
10 substitution are leucine, isoleucine, tyrosine, phenylalanine, tryptophan, valine, methionine, cysteine, alanine, histidine, proline, threonine, glycine, asparagine, and glutamine. The more preferred amino acid residues that may be substituted for  $X_2$  are cysteine, phenylalanine,  
15 glycine, isoleucine, leucine, methionine, valine, and tyrosine. Among the more preferred amino acid residues mentioned above, the even more preferred amino acid residues for substitution for  $X_2$  are leucine, isoleucine, tyrosine, valine, and methionine. The most preferred amino acid  
20 residue for substitution for  $X_2$  is leucine.

Additionally, as discussed above,  $X_3$  and  $X_4$  may be selected from all amino acid residues, but the preferred amino acid residues are valine, isoleucine, tyrosine, phenylalanine, tryptophan, methionine, cysteine, alanine,  
25 histidine, proline, threonine, glycine, glutamine, asparagine, and serine. The more preferred amino acid residues that may be substituted for  $X_3$  and  $X_4$  are valine, isoleucine, leucine, tyrosine, phenylalanine, methionine, cysteine, alanine, histidine, and proline. Among the more  
30 preferred amino acid residues mentioned above, the even more preferred amino acid residues that may be substituted for  $X_3$  and  $X_4$  are valine, isoleucine, leucine, methionine, and cysteine. Even more preferable for substitution for  $X_3$  and  $X_4$  are isoleucine and valine. The most preferred amino acid  
35 residue for substitution for  $X_3$  and  $X_4$  is valine. Also,  $X_5$  may be selected from all amino acid residues, but the preferred amino acid residues are alanine, histidine,



cysteine, methionine, valine, leucine, isoleucine, tyrosine, phenylalanine, proline, threonine, glycine, glutamine, asparagine, and serine. The more preferred amino acid residues that may be substituted for X<sub>5</sub> are alanine, histidine, cysteine, methionine, valine, proline, threonine, glycine, glutamine, asparagine, and serine. Even more preferred amino acid residues for substitution for X<sub>5</sub> are alanine, histidine, cysteine, proline, threonine, glycine, glutamine, asparagine, and serine. The most preferred amino acid residue for substitution for X<sub>5</sub> is alanine.

Moreover, X<sub>6</sub> may be selected from all amino acid residues, but the preferred amino acid residues for substitution are threonine, proline, alanine, histidine, cysteine, methionine, valine, leucine, isoleucine, tyrosine, glycine, glutamine, asparagine, and serine. The more preferred amino acid residues for substitution for X<sub>6</sub> are threonine, proline, alanine, histidine, cysteine, methionine, glycine, glutamine, asparagine, and serine. Even more preferred amino acid residues for substitution are threonine, proline, alanine, histidine, and glycine. The most preferred amino acid residue for substitution for X<sub>6</sub> is threonine. Also, X<sub>7</sub> may be selected from all amino acid residues, but the preferred amino acid residues are glycine, threonine, proline, alanine, histidine, cysteine, methionine, valine, leucine, isoleucine, glutamine, asparagine, serine, glutamic acid, and aspartic acid. The more preferred amino acid residues for substitution for X<sub>7</sub> are glycine, threonine, proline, alanine, histidine, cysteine, glutamine, asparagine, and serine. Even more preferred amino acid residues for substitution for X<sub>7</sub> are glycine, threonine, proline, alanine, histidine, glutamine, asparagine, and serine. The most preferred amino acid residue for substitution for X<sub>7</sub> is glycine.

The polypeptides of the present invention can be combined with various excipient vehicles and/or adjuvants well known in this art which serve as pharmaceutically acceptable carriers to permit drug administration in the

form of, e.g., injections, suspensions, emulsions, tablets, capsules, and ointments. These pharmaceutical compositions may be administered by any acceptable means. For warm-blooded animals, and in particular, for humans,

5 administration can be oral, parenteral, subcutaneous, intravenous, intramuscular and/or intraperitoneal. The specific dose administered will be dependent upon such factors as the general health and physical condition of the subject as well as the subject's age and weight, the stage

10 of the subject's disease condition, the existence of any concurrent treatments, and the frequency of administration; typically, the dose will be in the range of about 0.5 to about 2.0 mg/kg for human subjects. In general, the composition will contain one or more of the polypeptide(s)

15 of the present invention in a concentration of at least about 0.0001% by weight, more typically at least about 0.001% by weight, still more typically at least about 0.01%, still more typically at least about 0.1% and, in some embodiments, in a concentration of at least about 1% by

20 weight of the composition.

Human uroguanylin cDNA has been cloned in bacteria, and chemically synthesized by solid phase peptide synthesis. Uroguanylin peptide can be chemically synthesized by using the procedure as described in U.S. patent number 5,489,670

25 *Human Uroguanylin* and in U.S. patent number 5,140,102 *Pentadecapeptide, guanylin, which stimulates intestinal guanylate cyclase*. Peptides similar to uroguanylin peptides have been identified in mouse, rat, porcine, and bovine species. The functionally active domain in most of these

30 peptides are highly conserved. Therefore, the physiological functions of these peptides may be similar, and these peptides may be used as intestinal cancer preventative agents as well. Thus, as long as the functionally active domains of these peptides are conserved, substitutions in

35 the non-active domains may be achieved with no change in the activity of the peptides.

All references, patents or applications U.S. or foreign, cited in the application are hereby incorporated by reference as if written herein.

In order to further illustrate the invention, the following exemplary laboratory preparative work was performed. However, it will be appreciated that the invention is not limited to these examples or the details described therein.

#### EXAMPLES

##### 10        Materials and Methods

Cell Culture. T-84 cells were obtained from the American Type Culture Collection at passage 52. Cells were grown in 1:1 mixture of Ham's F-12 medium and Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100U penicillin/ml, and 100 µg/ml streptomycin. Cells were fed fresh medium every third day and split at a confluence of approximately 80%.

Human tissue. Samples of normal colon and tumors were obtained following colon resections for adenocarcinoma under a human experimentation protocol that was approved by the Missouri University/Truman VA Hospital Committee. Mucosa samples from normal colon tissues adjacent to the colon adenocarcinomas were isolated from submucosal tissue by scraping the mucosal surface with a microscope slide to separate mucosa from the underlying tissue. Portions of the tumors were collected and processed as an intact tissue. Tissues from eleven subjects between the ages of 48 and 82 years representing female and male patients were used in this study.

**EXAMPLE 1**Materials and Methods

Cell proliferation assay. Approximately 10,000 T-84 cells were inoculated in each well of 96-well plates. After  
5 an incubation period of 3 days, the indicated concentrations of human uroguanylin were added to the media and cells were allowed to grow until they formed semi-confluent monolayers. Subsequently, BrdU labeling agent (5-bromo-2'-deoxyuridine in PBS) was added (final concentration 100  $\mu$ M) and cells  
10 were re-incubated for an additional 24 hours. Monolayers were washed and incorporation of BrdU was measured following the manufacturer's instructions (Boehringer Mannheim Corp., Indianapolis, IN).

Results:

15 Uroguanylin treatment caused a dose-dependent inhibition in growth of these cells, reaching an approximately 30% growth inhibition at 1  $\mu$ M as seen in figure 2. In contrast, a biologically inactive variant of this peptide did not inhibit cell growth suggesting that the  
20 growth inhibition was a receptor-mediated event.

**EXAMPLE 2**Materials and Methods

Apoptosis assay. T-84 cells were grown in 35 mm dishes for 7 days. The confluent monolayers were washed once with  
25 serum-free DMEM, and incubated with the same media containing different concentrations of human uroguanylin for 16 hours. After this incubation, cells were quickly collected by trypsinization, and the cell pellet was washed twice with phosphate buffer saline (PBS). Cells were  
30 resuspended in PBS at a concentration of approximately  $10^8$  cell/ml. For demonstration of nucleosomal ladders, the apoptotic DNA was isolated from these cells by following the instructions of the DNA fragmentation analysis kit (Boehringer Mannheim Corp., Indianapolis, IN). The  
35 apoptotic DNA was separated by agarose gel electrophoresis followed by staining with ethidium bromide. Induction of

apoptosis by uroguanylin was further demonstrated by using the TUNEL assay as per the instructions of the 'In situ cell death detection kit' (Boehringer Mannheim Corp., Indianapolis, IN).

5        Results:

As shown in figure 3, the DNA isolated from the control (lane 2) as well as from the biologically inactive variant of uroguanylin treated cells (lane 6) exhibited very low levels of DNA fragmentation, consistent with a low basal  
10 rate of apoptosis under serum-free conditions. On the other hand, DNA from the uroguanylin treated cells exhibited extensive DNA fragmentation in a dose-dependent manner. The induction of apoptosis by uroguanylin treatment was further supported by the terminal deoxynucleotidyl transferase-  
15 mediated dUTP-biotin nick end labeling (TUNEL) assay using CaCo-2 cells. Uroguanylin treatment significantly augmented the generation of apoptotic cells compared to the vehicle treated cells as seen in figure 4. These results confirmed that uroguanylin induces apoptosis in human colon cancer  
20 cells (T-84 and CaCo-2).

EXAMPLE 3

Uroguanylin functional assay. Human uroguanylin (NDDCELCVNVACTGCL) peptide was custom synthesized by Multiple Peptide System, San Diego, CA. The biological  
25 activity of the synthetic peptide was assayed by a modified cell-based assay. Briefly, the confluent monolayers of T-84 cells in 24-well plates were washed twice with 250  $\mu$ l of DMEM containing 50 mM HEPES (pH 7.4), preincubated at 37° C for 10 min with 250  $\mu$ l of DMEM containing 50 mM HEPES (pH  
30 7.4) and 1 mM isobutylmethylxanthine (IBMX), followed by incubation with different concentration of human uroguanylin ( $10^{-6}$  to  $10^{-10}$  M) for 30 min. The medium was aspirated, and the reaction was terminated by the addition of 3% perchloric acid. The plate was centrifuged at 1000xg for 5 min and the  
35 supernatant was collected. After neutralization with 0.1N NaOH, the supernatant was used directly for measurements of

cGMP by using the ELISA kit from Caymen Chemical, Ann Arbor, MI. Results are expressed as an average of three determinations.

Results:

- 5        Biological activity was observed in several isoforms of this peptide as indicated by the cGMP levels observed.

**EXAMPLE 4**

Ussing chamber assay. The seromuscular layer of human intestinal mucosa was removed by blunt dissection and one to  
10    four mucosal sheets from each specimen (~1cm<sup>2</sup>) were used. To collect intestinal mucosa from mice, animals were sacrificed by 100% CO<sub>2</sub> inhalation. A mid-line abdominal incision was used to excise the intestinal mucosal layer. The dissected intestinal tissue was opened along the mesenteric border in  
15    ice-cold, oxygenated Krebs-Ringer-bicarbonate (KRB) solutions and pinned luminal-side down on a pliable silicone surface. The outer muscle layers were striped by shallow dissection with a scalpel and fine forceps. Mouse intestine and human colon tissue, consisting of mucosa and submucosa,  
20    were mounted between two ussing half-chambers and bathed on both sides. Electrical measurements were monitored with an automatic voltage clamp, and direct-connecting voltage and current passing difference and  $I_{sc}$ . Tissues were equilibrated under short-circuit conditions until  $I_{sc}$  had  
25    stabilized and the potential difference across the epithelium was measured intermittently.

Human uroguanylin peptide was chemically synthesized and the relative potencies of various synthetic forms were evaluated by their abilities to stimulate cGMP accumulation  
30    in intact T-84 cells. The biological activity of several isoforms of this peptide that exhibited similar physico-chemical properties was observed. The major isoform, exhibiting a potent biological activity, was further purified to about 99% purity and used for this study. To  
35    ensure that the synthetic form of human uroguanylin was equally effective in mouse and human GI mucosa, its activity

was examined in the Ussing chamber using the mouse duodenum and the human colonic mucosa.

Results:

The pattern of  $I_{sc}$  responses to sequential treatment with specific agents on the mouse duodenum (Fig. 1a) and human colon (Fig. 1b) were recorded. In both tissues, the addition of TTX ( $0.1 \mu\text{M}$  in serosal bath) resulted in a decrease in the baseline  $I_{sc}$  to a stable value within 20 minutes. Subsequent addition of uroguanylin ( $0.1 \mu\text{M}$  in the luminal bath) resulted in a rapid increase in  $I_{sc}$ , which was sustained for a 60 minute period. Carbachol, a known stimulator of ion transport across the membrane, further increased the  $I_{sc}$ . These results confirmed that the synthetic human uroguanylin peptide was effective in stimulating electrolytes transport in human as well as in mouse intestinal mucosa.

**EXAMPLE 5**

Methods and Materials

Min-mouse model. Male Min mice (C57BL/6J-APC<sup>Min</sup>/+), a strain containing a fully penetrant dominant mutation in the APC gene, were obtained at 4-5 weeks of age from The Jackson Laboratory, Bar Harbor, ME. All mice were fed a high-fat AIN-93G diet, tap water to drink and housed in a humidity and temperature controlled room with a 12 hour light-dark cycle. Animals consumed approximately 5 grams of the diet per day. After one week of quarantine period, animals were randomly divided in three groups of 10 animals each. These groups of animals were fed the same diet containing different concentrations of (0, 10, and  $20 \mu\text{g}/5\text{grams}$  of the diet) of human uroguanylin. Animals were also given additional amounts of human uroguanylin (vehicle, 10 and  $20 \mu\text{g}$ ) in 0.2 ml of PBS containing 20% polyethylene glycol by oral gavage twice a week. Food consumption and body weight of these animals were monitored weekly. At the end of the 17th week, animals were sacrificed by  $\text{CO}_2$  asphyxiation and the GI tracts were removed. After flushing with PBS to

remove food materials, the GI tract was divided as sections of duodenum (two (2) inches from the stomach), jejunum (middle portion, approx. 4-5 inches from the stomach), ileum (two (2) inches from the cecum) and colon. These sections  
5 were opened longitudinally, washed with tissue fixative (Streck Laboratories, Inc., Omaha, NE) and placed between two layers of blotting paper in a tray containing the tissue fixative. Polyps and tumors were counted independently by four different observers. Results are expressed as the  
10 average of the total number of polyps for each individual animal by four different observers. Analysis of the data obtained from all observers revealed insignificant inter-observer variance. Sections of these tissues were viewed under a constant magnification (10 X) to gauge the  
15 differences between polyp diameter between animals.

#### Results:

The Min-mouse, the most widely used animal model to assess the chemopreventive properties of dietary nutrients and therapeutic agents, carries a dominant mutation in one  
20 of the alleles of the APC gene. Thus, when these mice are raised on a high fat diet, they begin to develop polyps throughout the intestine at around 55 days of age. Development of polyps causes blockage in the movement of intestinal contents, which leads to decreased food  
25 consumption and reduced gain in body weight as the disease progresses. The test results for oral administration of uroguanylin to the min-mouse showed a dose-dependent increase in the food consumption as shown in figure 6a, and in the body weight gain as shown in figure 6b. The average  
30 body weight for the control group was  $25.1 \pm 0.9$  g, and that for the uroguanylin-treated ( $20\mu\text{g}$ ) group was  $29.4 \pm 1.07$  g at 17. In addition, animals treated with uroguanylin were visibly more healthy and active.

At the end of the study, all animals were sacrificed  
35 and the GI tract was removed to determine the number and distribution of polyps in the small intestine and colon tissues as shown in table I. The GI tract in the untreated



control group contained  $48.3 \pm 7.7$  polyps per mouse. A majority of the polyps were located throughout the small intestine and only a few polyps were found in the colon. The sizes of the polyps in the control group of mice were in the range of approximately 3 to 5 mm. Three animals in the control group had also developed globular tumors in the duodenum. Administration of uroguanylin reduced the total number of polyps ( $23.3 \pm 3.1$ ) by approximately 50%. In addition, polyps in uroguanylin-treated group of mice were significantly smaller in size ( $<2.0$  mm). There were no polyps observed in the colons of any animals in this group, nor were there any globular tumors in these animals. Since the appearance of polyps in colon of Min-mice occurs only during the severe cases of diseases, the absence of polyps in colon of the uroguanylin-treated group of mice suggest that this peptide might also inhibit the progression of colon cancer. These results suggest that the oral administration of uroguanylin suppresses both the formation as well as the progression of polyp formation in this animal model for colon cancer.

TABLE 1: Inhibition of Polyps formation in Min-mouse by oral administration of human uroguanylin.

Treatments	Average Numbers of Polyps/mouse				Total	Remarks
	Duodenum	Jejunum	Ileum	Colon		
Vehicle Control Group A*	6.4 ± 0.8	26.9 ± 5.4	14.3 ± 2.6	0.8 ± 0.2	48.3 ± 7.7	Three animals showed globular tumor in the duodenum. The average size of polyps was in the range of 3.0 to 5.0 mm.
Uroguanylin Group B	2.0 ± 0.5	21.2 ± 4.2	8.7 ± 1.6	0.2 ± 0.1	32.1 ± 5.2	The average size of polyps was 2.0-3.0 mm.
Uroguanylin Group C	2.2 ± 0.6	12.5 ± 1.6	8.7 ± 1.8	0.0	23.3 ± 3.1**	Polyps were <2.0 mm size.

All results are shown as mean ± SEM, n=10.

\*Uroguanylin was administered by p.o. twice a week: Group A, vehicle control; group B, 10 µg uroguanylin and Group C, 20 µg.

\*\*p<0.05 compared with the control group.

## EXAMPLE 4

Expression of Uroguanylin in human colon cancer tissues.

Methods and Materials

5 Isolation of RNA. RNA was extracted from tissue using a combination of the TRI reagent method (Molecular Research Center, Inc., Cincinnati, OH) and the RNAeasy Kit (Qiagen, Valencia, CA). The tissue was homogenized in TRI reagent following the manufacturer's protocol. After phase  
10 separation with chloroform, the aqueous supernatant phase containing total RNA was removed and mixed with an equal volume of 70% ethanol and lysis buffer without beta-mercaptoethanol. The resulting mixture was loaded onto the RNAeasy columns and then processed following the protocol  
15 provided by the manufacturer.

Northern blotting. Total RNA (20  $\mu$ g) was subjected to electrophoresis in formaldehyde-agarose gels and then transferred to nylon membranes (Zeta-Probe, Bio-Rad Laboratories, Inc., Hercules, CA). The membranes were  
20 prehybridized for two hours at 65°C in ExpressHyb solution (Clontech, Palo Alto, CA) and then hybridized with human guanylin, uroguanylin and GC-C cDNAs overnight at 65°C. All cDNA probes were labeled with  $^{32}$ P by random priming (Boehringer Mannheim, Indianapolis, IN). RNA blots were  
25 then washed twice with 2X SSC-0.1% SDS for 5 min at room temperature followed by a 15 min wash at 60°C with 0.2X SSC-0.1% SDS. Exposure to X-ray film was performed at -80°C with intensifying screens.

RT-PCR. Oligo(deoxythymidine)<sub>18</sub>-primed cDNAs were  
30 synthesized from 3  $\mu$ g total RNA using reverse transcriptase (Superscript II, Life Technologies, Gaithersburg, MD). Two PCR primers, 5'-primer (5'-GAACCCAGGGAGCGCGAT-3') [identified as SEQ. ID. 6] and 3'-primer (5'-CTGGTGGGCTCAGGGTACC-3') [identified as SEQ. ID 7], were designed from regions  
35 flanking the open reading frame of human pre-prouroguanylin cDNA. A PCR product of the expected size of 384 bp was

amplified from colon cDNAs after 25 cycles at 93°C for 1 min, 56°C for 1 min, and 72°C for 1.5 min using Taq DNA polymerase (U.S. Biochemical Corp., Cleveland, OH). The pair of primers for RT-PCR of guanylin were 5'-primer (5'-  
5 AACTCAGGAACTTTGCAC-3') [identified as SEQ. ID. 8] and 3'-  
primer (5'-CGTAGGCACAGATTTCAC-3') [identified as SEQ. ID. 9]. These primers produced a 174 bp cDNA for human guanylin using the PCR conditions of 25 cycles at 93°C for 1 min, 59°C for 1 min and 72°C for 1.5 min. The PCR-generated cDNA  
10 products were subjected to electrophoresis on 1% agarose gels in TAE buffer containing ethidium bromide and then transferred to nylon membranes. Southern hybridization was carried out using the uroguanylin and guanylin cDNA probes. Prehybridization was for 1 hour at 65°C with ExpressHyb  
15 solution and then hybridization was for 3 hours at 65°C. Blots were washed as described above and exposed to X-ray films at -80°C with intensifying screens.

Results:

The expression of uroguanylin, guanylin and GC-C receptor in eleven samples of human colon carcinoma and the surrounding normal tissues. Northern blot analysis showed  
5 that the expression of uroguanylin and guanylin was completely suppressed in all specimens examined, whereas the adjacent non-cancerous tissue from the same patient exhibited a robust expression of these transcripts as shown in figure 5a. A similar expression pattern was observed  
10 when these tissue specimens were analyzed by a more sensitive RT-PCR followed by Southern blotting based analysis as shown in figure 5b. Despite the fact that these specimens were from different stages of colon cancer and were from a different age group of patients, the expression  
15 of guanylin and uroguanylin was severely suppressed in all eleven tissue specimens examined. These results raise the possibility that the loss of these intestinal hormones either leads to or is a result of adenocarcinoma formation.

In light of the detailed description of the invention  
20 and the examples presented above, it can be appreciated that the several objects of the invention are achieved.

The explanations and illustrations presented herein are intended to acquaint others skilled in the art with the invention, its principles, and its practical application.  
25 Those skilled in the art may adapt and apply the invention in its numerous forms, as may be best suited to the requirements of a particular use. Accordingly, the specific embodiments of the present invention as set forth are not intended as being exhaustive or limiting of the invention.

## WHAT IS CLAIMED IS:

1. A method of modulating polyps in the intestine of a subject, the process comprising administering to the subject, in need thereof, a pharmaceutical composition comprising a polypeptide having the sequence:

5

$X_8$ -Asp -Asp -Cys - $X_1$  - $X_2$ -Cys - $X_3$  -Asn - $X_4$  - $X_5$  -Cys - $X_6$  - $X_7$  -Cys- $X_9$

wherein each of  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$ ,  $X_5$ ,  $X_6$ , and  $X_7$  is an amino acid residue,  $X_8$  and  $X_9$  are independently hydrogen or at least one amino acid residue, and the polypeptide is cross-linked by a  
10 disulfide bond between the cystine residue immediately adjacent the amine group of  $X_1$  and the cystine residue immediately adjacent the amine group of  $X_6$  and by a disulfide bond between the cystine residue immediately adjacent the  
15 amine group of  $X_3$  and the cystine residue immediately adjacent the carboxy group of  $X_7$ , together with a pharmaceutically acceptable carrier.

2. A method of modulating polyps in the intestine of a subject, the process comprising administering to the subject, in need thereof, a pharmaceutical composition comprising an agonist peptide or compound which binds to the  
5 guanylate cyclase receptor GC-C in the intestine of the subject, together with a pharmaceutically acceptable carrier.

3. A method of claim 1 wherein the concentration of the peptide in the composition is at least 0.0001 percent by weight of the composition.

4. A method of claim 1 wherein the concentration of the peptide in the composition is at least 0.001 percent by weight of the composition.

5. A method of claim 1 wherein the concentration of the peptide in the composition is at least 0.01 percent by weight of the composition.

6. A method of claim 1 wherein the concentration of the peptide in the composition is at least 0.1 percent by weight of the composition.

7. A method of claim 1 wherein the concentration of the peptide in the composition is at least 1 percent by weight of the composition.

8. The method of claim 1 wherein said subject has been determined to have a genetic predisposition for the growth of polyps in the intestine.

9. The method of claim 1 wherein polyps have been identified in the intestine of said subject.

10. The method of claim 1 wherein said subject has been identified as having intestine cancer.

11. A method of claim 2 wherein the concentration of the peptide in the composition is at least 0.0001 percent by weight of the composition.

12. A method of claim 2 wherein the concentration of the peptide in the composition is at least 0.001 percent by weight of the composition.

13. A method of claim 2 wherein the concentration of the peptide in the composition is at least 0.01 percent by weight of the composition.

14. A method of claim 2 wherein the concentration of the peptide in the composition is at least 0.1 percent by weight of the composition.

15. A method of claim 2 wherein the concentration of the peptide in the composition is at least 1 percent by weight of the composition.

16. The method of claim 2 wherein said subject has been determined to have a genetic predisposition for the growth of polyps in the intestine.

17. The method of claim 2 wherein polyps have been identified in the intestine of said subject.

18. The method of claim 2 wherein said subject has been identified as having intestine cancer.

19. The method of claim 1 wherein  $X_1$  is selected from a group of amino acid residues consisting of aspartic acid, glutamic acid, glycine, lysine, asparagine, proline, glutamine, arginine, serine, and threonine.

20. The method of claim 1 wherein  $X_1$  is selected from a group of amino acid residues consisting of glutamic acid, arginine, lysine, serine, aspartic acid, asparagine, glutamine, and glycine.

21. The method of claim 1 wherein  $X_1$  is selected from a group of amino acid residues consisting of glutamic acid, aspartic acid, arginine, and lysine.

22. The method of claim 1 wherein  $X_1$  is glutamic acid.

23. The method of claim 1 wherein  $X_2$  is selected from a group of amino acid residues consisting of leucine, isoleucine, tyrosine, phenylalanine, tryptophan, valine, methionine, cysteine, alanine, histidine, proline,  
5 threonine, glycine, asparagine, and glutamine.



24. The method of claim 1 wherein  $X_2$  is selected from a group of amino acid residues consisting of cysteine, phenylalanine, glycine, isoleucine, leucine, methionine, valine, and tyrosine.

25. The method of claim 1 wherein  $X_2$  is selected from a group of amino acid residues consisting of leucine, isoleucine, tyrosine, valine, methionine.

26. The method of claim 1 wherein  $X_2$  is selected from a group of amino acid residues consisting of leucine, and isoleucine.

27. The method of claim 1 wherein  $X_2$  is leucine.

28. The method of claim 1 wherein  $X_3$  is selected from the group of amino acid residues consisting of valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, methionine, cysteine, alanine, histidine, proline,  
5 threonine, glycine, glutamine, asparagine, and serine.

29. The method of claim 1 wherein  $X_3$  is selected from the group of amino acid residues consisting of valine, isoleucine, leucine, tyrosine, phenylalanine, methionine, cysteine, alanine, histidine, and proline.

30. The method of claim 1 wherein  $X_3$  is selected from the group of amino acid residues consisting of valine, isoleucine, leucine, methionine, and cysteine.

31. The method of claim 1 wherein  $X_3$  is valine.

32. The method of claim 1 wherein  $X_3$  is isoleucine.

33. The method of claim 1 wherein  $X_4$  is selected from the group of amino acid residues consisting of valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan,

methionine, cysteine, alanine, histidine, proline,  
5 threonine, glycine, glutamine, asparagine, and serine.

34. The method of claim 1 wherein  $X_4$  is selected from the group of amino acid residues consisting of valine, isoleucine, leucine, tyrosine, phenylalanine, methionine, cysteine, alanine, histidine, and proline.

35. The method of claim 1 wherein  $X_4$  is selected from the group of amino acid residues consisting of valine, isoleucine, leucine, methionine, and cysteine.

36. The method of claim 1 wherein  $X_4$  is valine.

37. The method of claim 1 wherein  $X_5$  is alanine, histidine, cysteine, methionine, valine, leucine, isoleucine, tyrosine, phenylalanine, proline, threonine, glycine, glutamine, asparagine, and serine.

38. The method of claim 1 wherein  $X_5$  is selected from the group of amino acid residues consisting of alanine, histidine, cysteine, methionine, valine, proline, threonine, glycine, glutamine, asparagine, and serine.

39. The method of claim 1 wherein  $X_5$  is selected from the group of amino acid residues consisting of alanine, histidine, cysteine, proline, threonine, glycine, glutamine, asparagine, and serine.

40. The method of claim 1 wherein  $X_5$  is alanine.

41. The method of claim 1 wherein  $X_6$  is selected from a group of amino acid residues consisting of threonine, proline, alanine, histidine, cysteine, methionine, valine, leucine, isoleucine, tyrosine, glycine, glutamine,  
5 asparagine, and serine.

42. The method of claim 1 wherein  $X_6$  is selected from a group of amino acid residues consisting of threonine, proline, alanine, histidine, cysteine, methionine, glycine, glutamine, asparagine, and serine.

43. The method of claim 1 wherein  $X_6$  is selected from a group of amino acid residues consisting of threonine, proline, alanine, histidine, and glycine.

44. The method of claim 1 wherein  $X_6$  is threonine.

45. The method of claim 1 wherein  $X_7$  is selected from a group of amino acid residues consisting of glycine, threonine, proline, alanine, histidine, cysteine, methionine, valine, leucine, isoleucine, glutamine, asparagine, serine, glutamic acid, and aspartic acid.

46. The method of claim 1 wherein  $X_7$  is selected from a group of amino acid residues consisting of glycine, threonine, proline, alanine, histidine, cysteine, glutamine, asparagine, and serine.

47. The method of claim 1 wherein  $X_7$  is selected from a group of amino acid residues consisting of glycine, threonine, proline, alanine, histidine, glutamine, asparagine, and serine.

48. The method of claim 1 wherein  $X_7$  is glycine.

49. The method of claim 1 wherein the polypeptide is uroguanylin.

50. The method of claim 1 wherein the polypeptide is human uroguanylin.

51. The method of claim 1 wherein the composition comprises pro-uroguanylin.

52. The method of claim 1 wherein the composition comprises human pro-uroguanylin.

53. The method of claim 2 wherein the composition comprises guanylin.

54. The method of claim 2 wherein the composition comprises lymphoguanylin.

55. The method of claim 2 wherein the composition comprises prolymphoguanylin.

56. The method of claim 2 wherein the composition comprises heat stable enterotoxin.

57. The method of claim 1 wherein the composition comprises a polypeptide, which is degraded with endogenous proteases of the subject, into uroguanylin.

58. The method of claim 1 wherein about 0.5 mg to about 2 mg of the polypeptide is administered per kilogram of the subject's weight.

59. The method of claim 1 wherein the subject is human.

60. The method of claim 2 wherein the composition comprises a polypeptide, which is degraded with endogenous proteases of the subject, into guanylin.

61. The method of claim 1 wherein  $X_1$  is glutamic acid,  $X_2$  is leucine,  $X_3$  is isoleucine,  $X_4$  is valine,  $X_5$  is alanine,  $X_6$  is threonine, and  $X_7$  is glycine.

62. A method for the prevention, inhibition and treatment of cancer in the intestine of a subject, the

process comprising administering to the subject the composition of claim 1.

63. A method for the prevention, inhibition and treatment of cancer in the intestine of a subject, the process comprising administering to the subject the composition of claim 2.

64. The method of claim 62 wherein the composition comprises uroguanylin.

65. The method of claim 63 wherein the composition comprises uroguanylin.

66. The method of claim 62 wherein the composition comprises pro-uroguanylin.

67. The method of claim 63 wherein the composition comprises pro-uroguanylin.

68. The method of claim 62 wherein the composition comprises human uroguanylin.

69. The method of claim 63 wherein the composition comprises human uroguanylin.

70. The method of claim 62 wherein the composition comprises human pro-uroguanylin.

71. The method of claim 63 wherein the composition comprises human pro-uroguanylin.

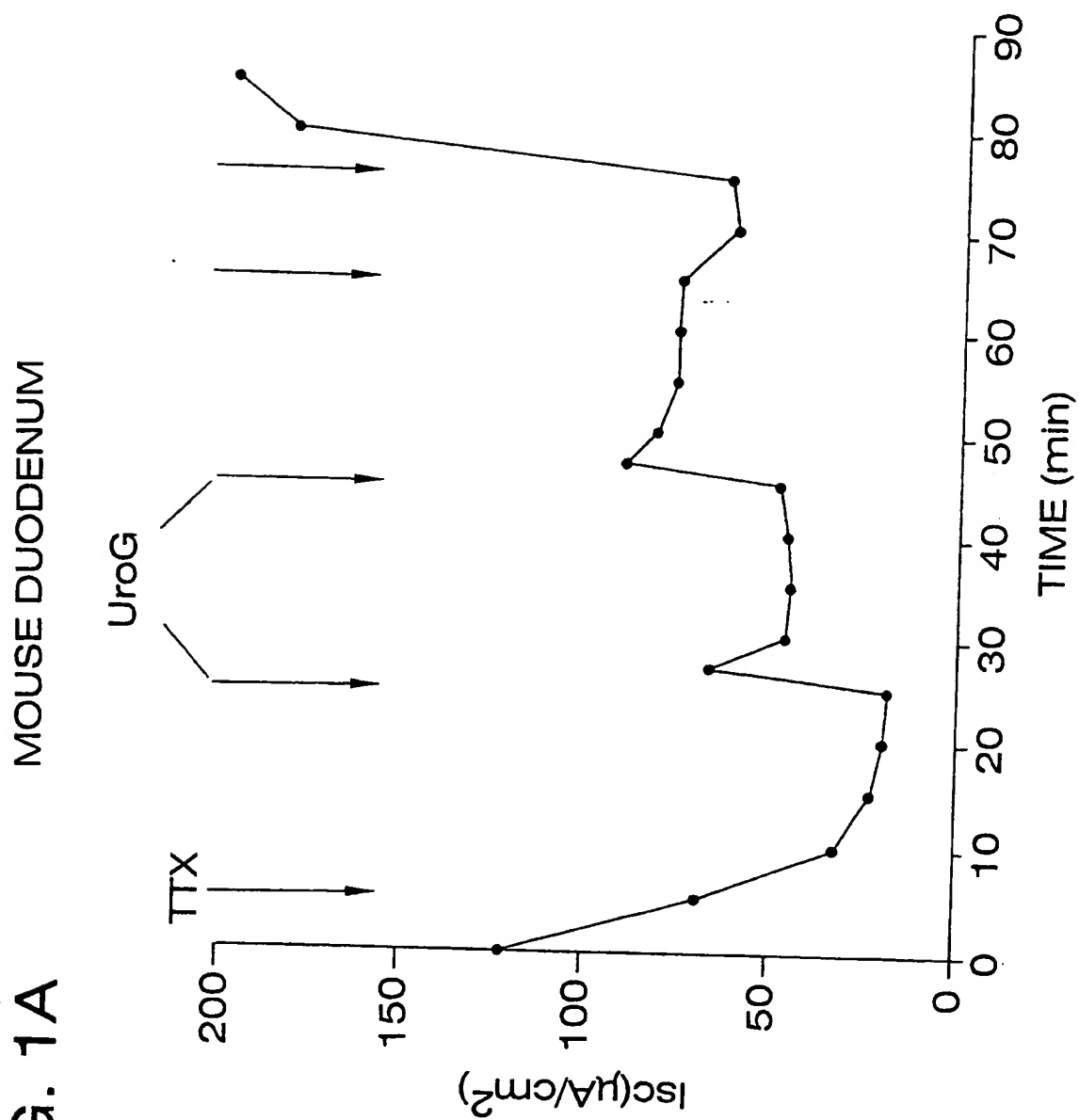
72. The method of claim 63 wherein the composition comprises guanylin.

73. The method of claim 63 wherein the composition comprises lymphoguanylin.

74. The method of claim 63 wherein the composition comprises heat stable enterotoxin.

75. The method of claim 63 wherein the composition comprises pro-lymphoguanylin.

FIG. 1A



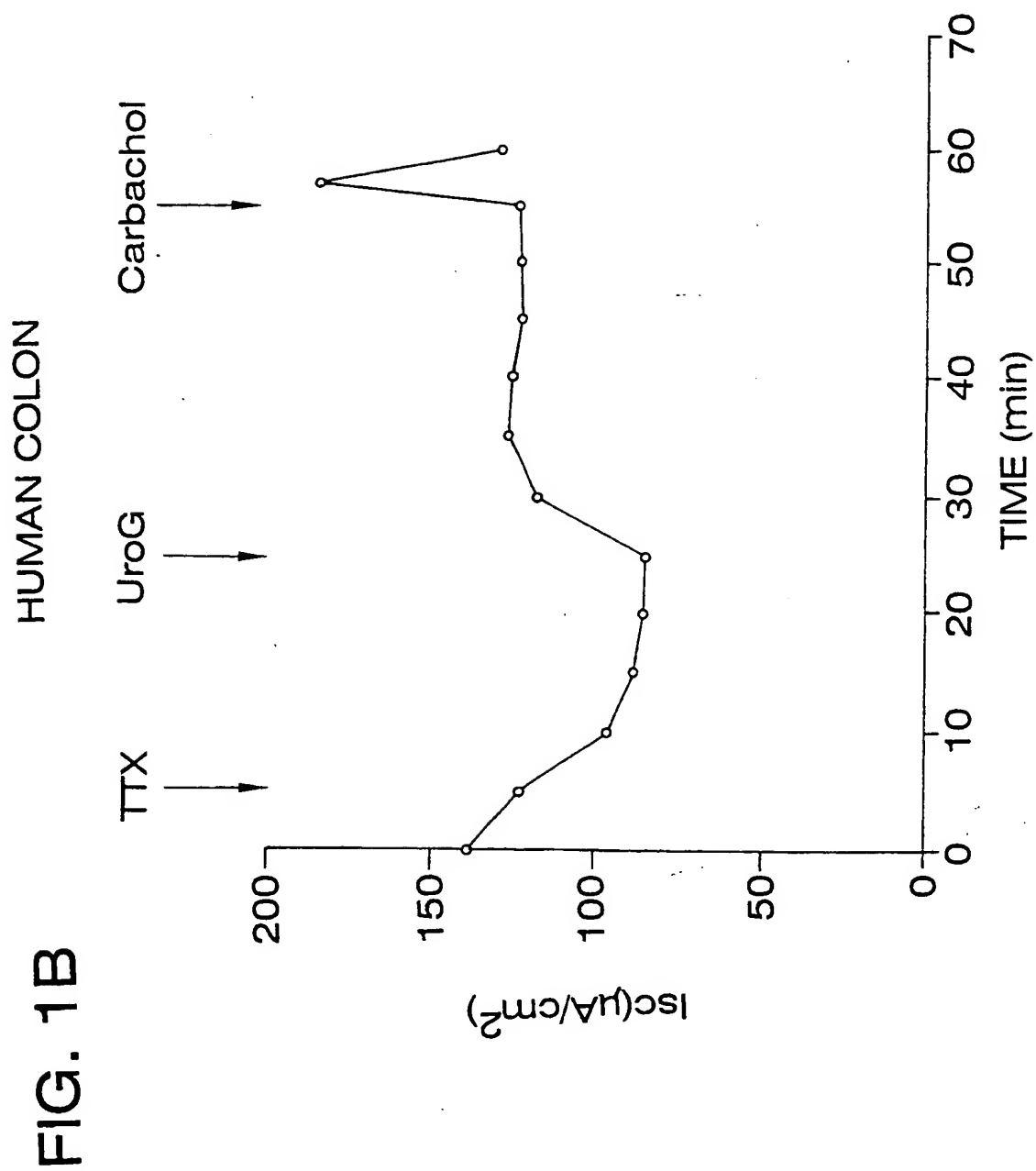




FIG. 2

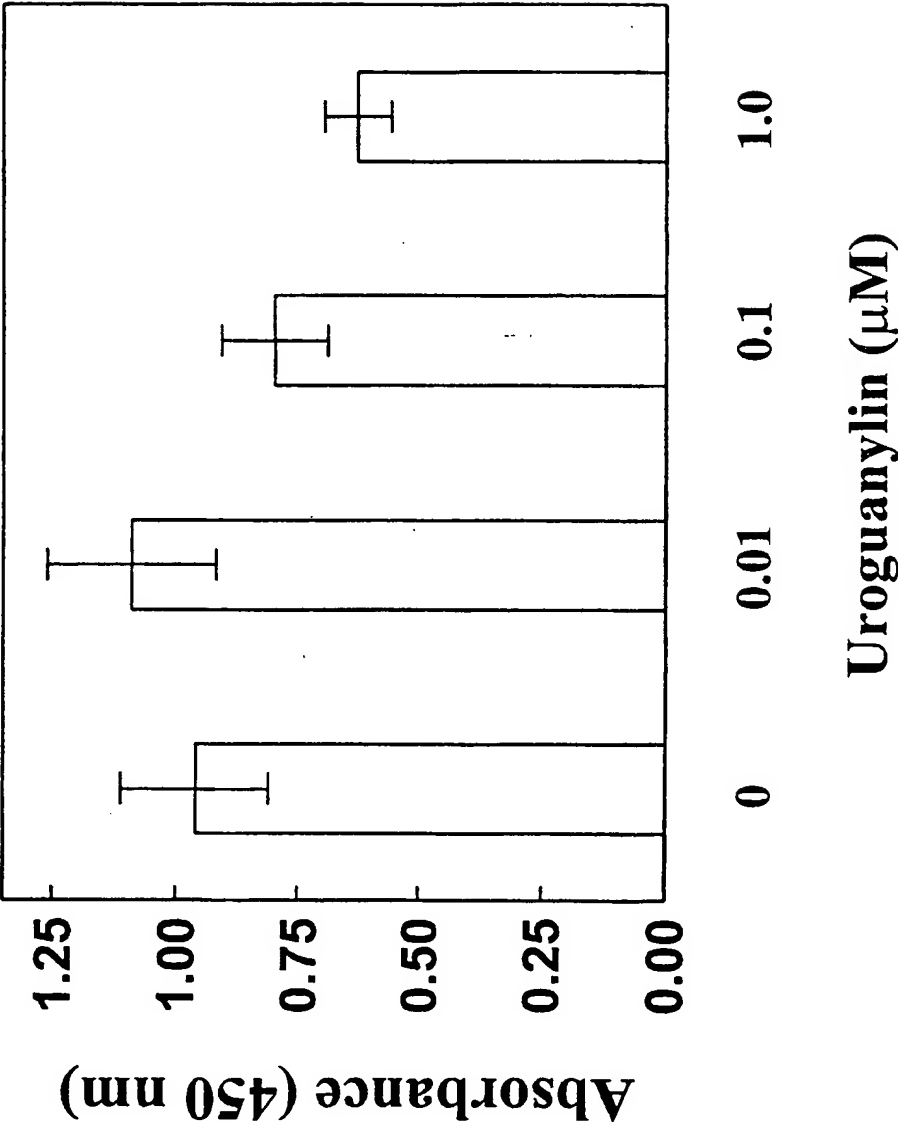


FIG. 3

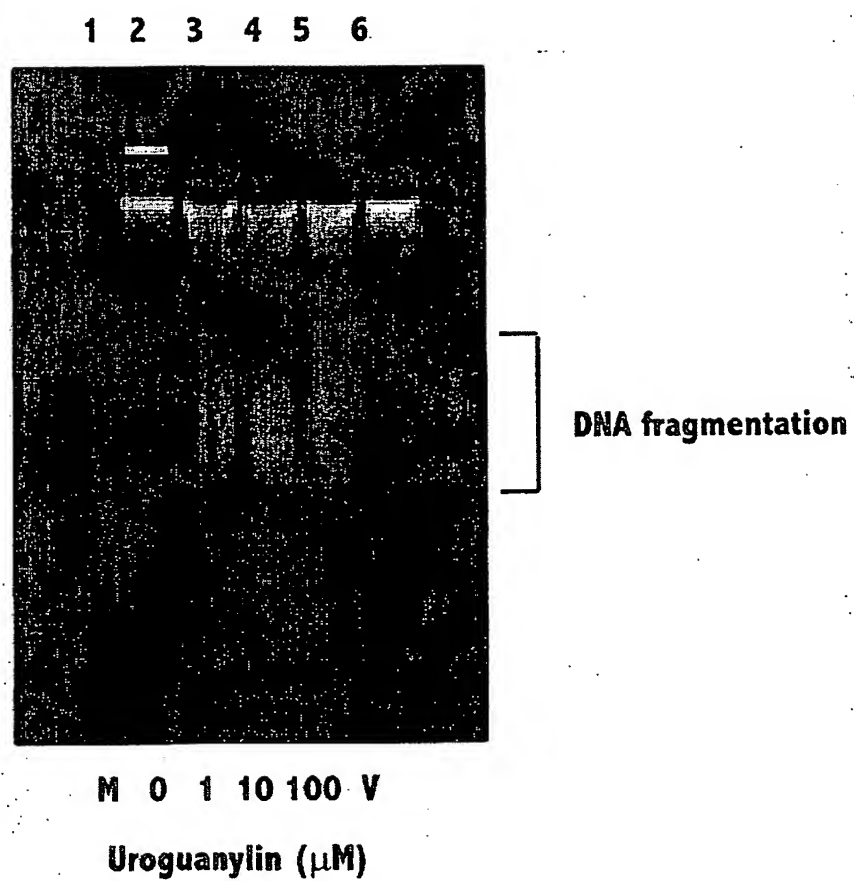


FIG. 4A

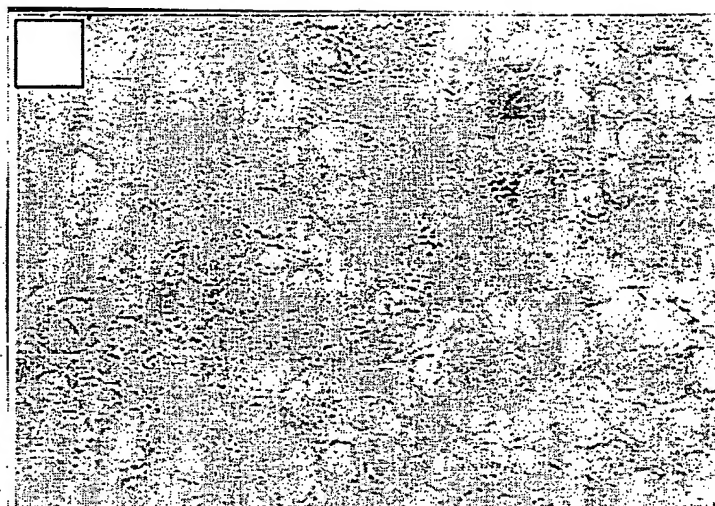


FIG. 4B

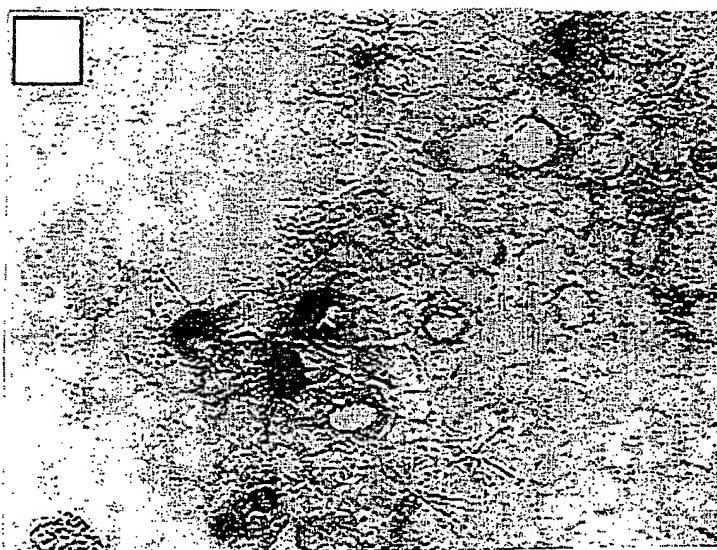
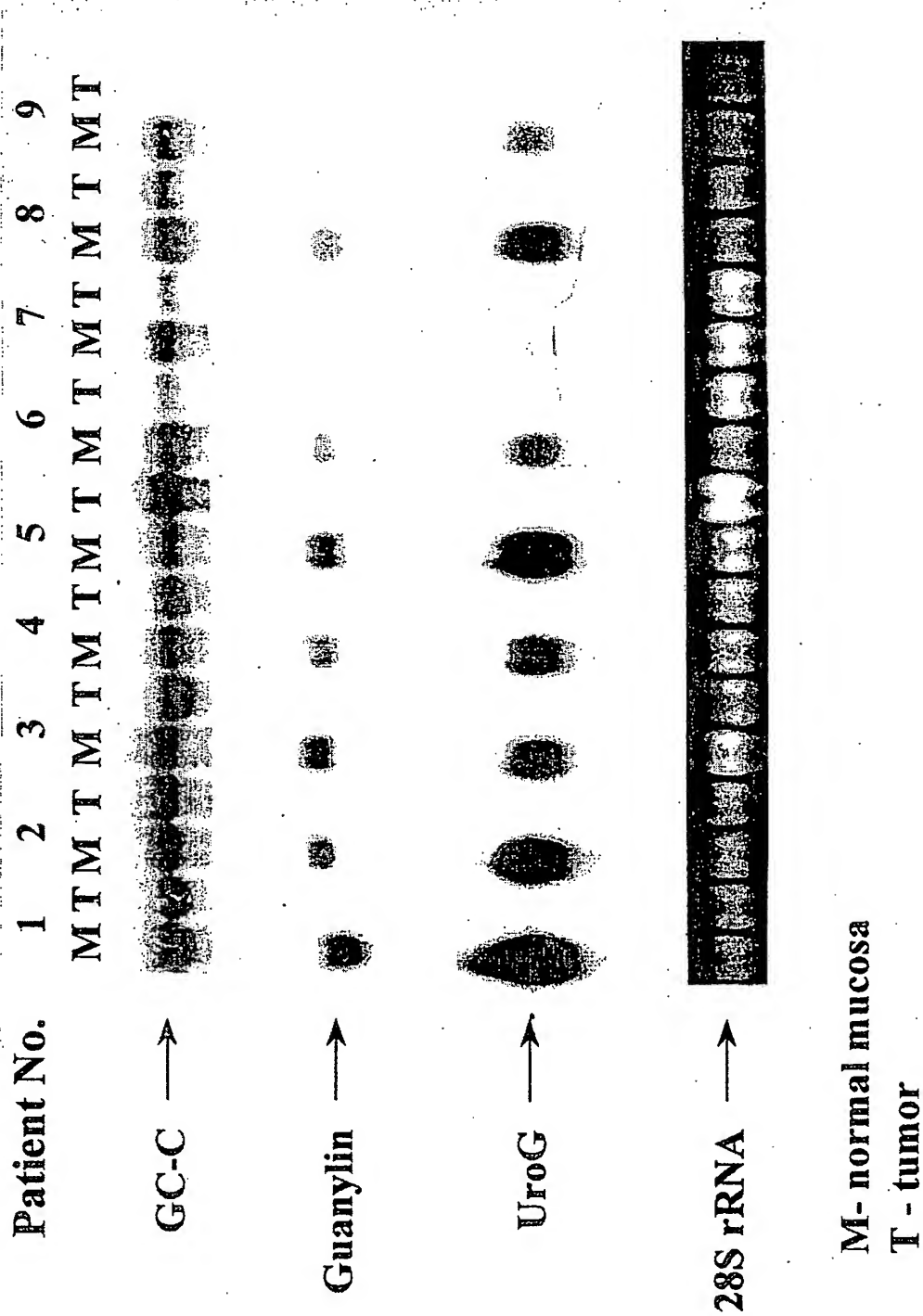


FIG. 5A





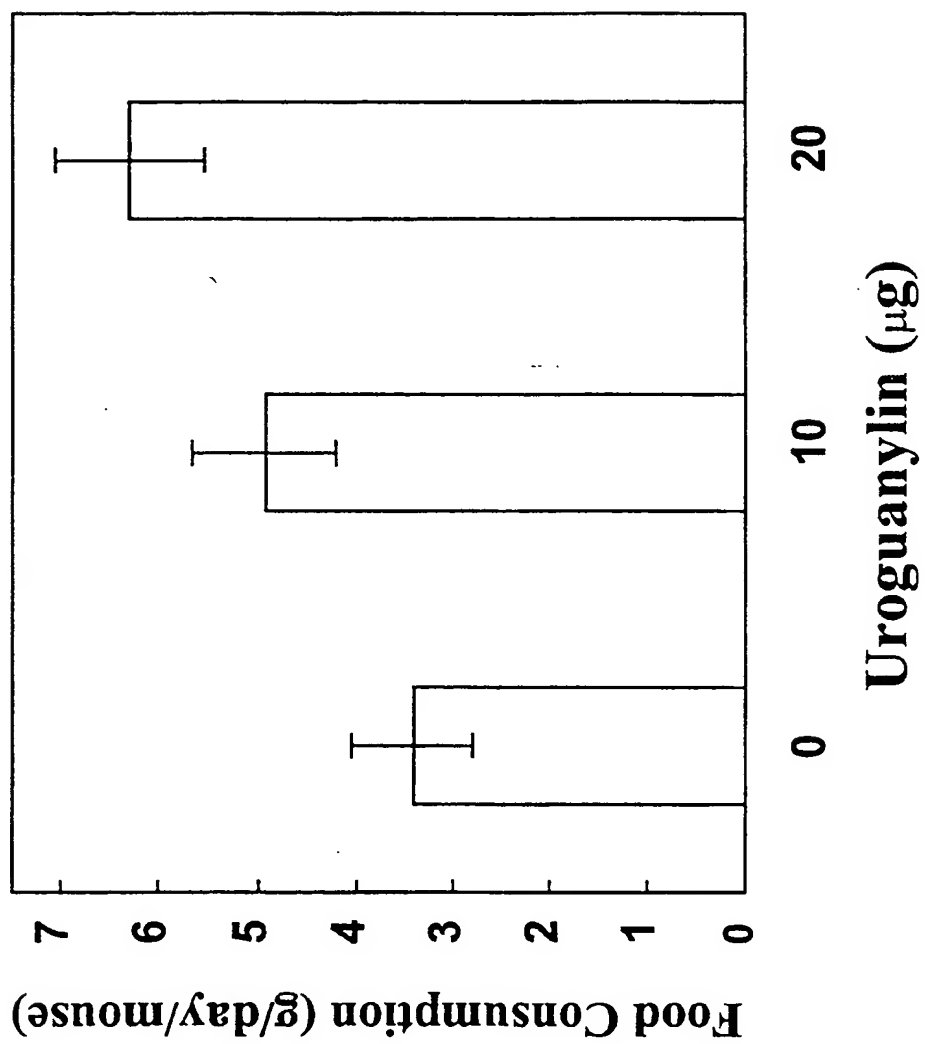


FIG. 6A

FIG. 6B

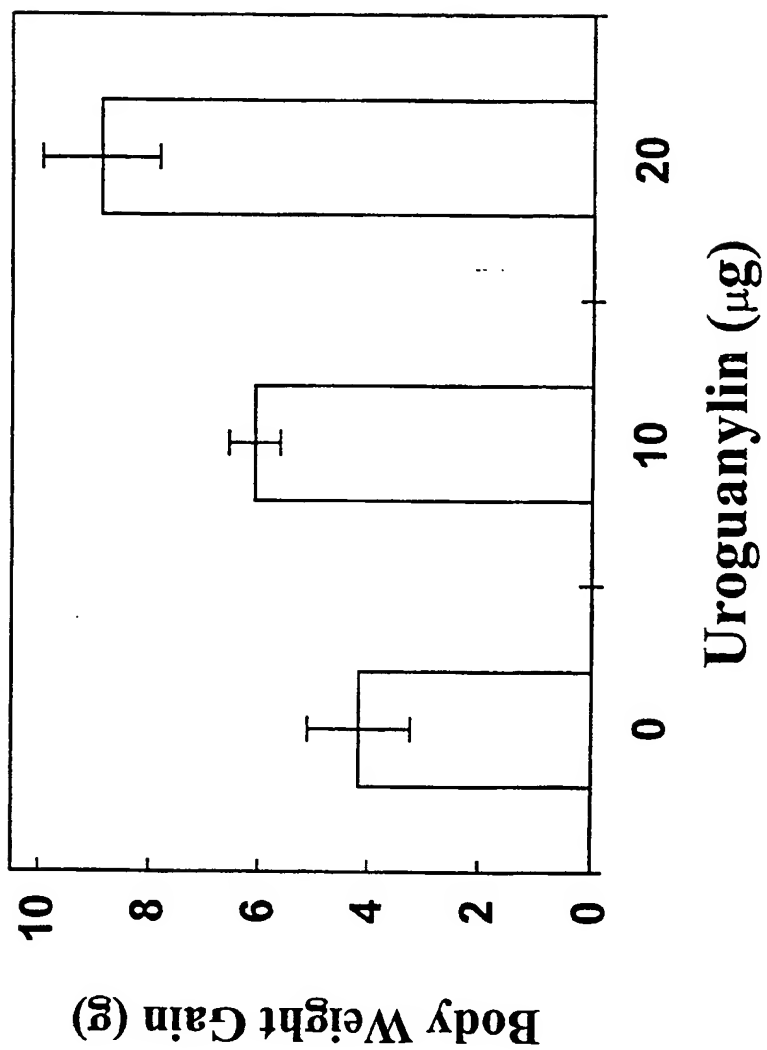


FIG. 7

Asn Asp Asp Cys *Glu* Leu Cys Val Asn Val Ala Cys Thr *Gly* Cys Leu h UroG.  
Pro Gly Thr Cys *Glu* Ile Cys Ala Tyr Ala Ala Cys Thr *Gly* Cys h Gua  
Asp Ser Ser Asn Tyr Cys Cys *Glu* Leu Cys Cys Asn Pro Ala Cys Thr *Gly* Cys Tyr E. coli  
Leu Ile Ile Asp Cys Cys *Glu* Ile Cys Cys Asn Pro Ala Cys Phe *Gly* Cys Leu Asn V. cholerae



## SEQUENCE LISTING

<110> Monsanto Company

<120> UROGUANYLIN AS AN INTESTINAL CANCER INHIBITING AGENT

<130> MTC6591.1

<140>

<141>

<150> US 60/157,950

<151> 1999-10-06

<160> 9

<170> PatentIn Ver. 2.1

<210> 1

<211> 16

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Polypeptide preferably of guanylan, uroguanylin, pro-uroguanylin, or another polypeptide which contains the active domain of uroguanylin

<220>

<221> VARIANT

<222> (1)

<223> X= Hydrogen or at least one amino acid residue

<220>

<221> VARIANT

<222> (5)

<223> X= any amino acid residue

<220>

<221> VARIANT

<222> (6)

<223> X= any amino acid residue

<220>

<221> VARIANT

<222> (8)

<223> X= any amino acid residue

&lt;220&gt;

&lt;221&gt; VARIANT

&lt;222&gt; (10)

&lt;223&gt; X= any amino acid residue

&lt;220&gt;

&lt;221&gt; VARIANT

&lt;222&gt; (11)

&lt;223&gt; X= any amino acid residue

&lt;220&gt;

&lt;221&gt; VARIANT

&lt;222&gt; (13)

&lt;223&gt; X= any amino acid residue

&lt;220&gt;

&lt;221&gt; VARIANT

&lt;222&gt; (14)

&lt;223&gt; X= any amino acid residue

&lt;220&gt;

&lt;221&gt; VARIANT

&lt;222&gt; (16)

&lt;223&gt; X= Hydrogen or at least one amino acid residue

&lt;400&gt; 1

Xaa	Asp	Asp	Cys	Xaa	Xaa	Cys	Xaa	Asn	Xaa	Xaa	Cys	Xaa	Xaa	Cys	Xaa
1				5					10					15	

&lt;210&gt; 2

&lt;211&gt; 16

&lt;212&gt; PRT

&lt;213&gt; Human uroguanylin

&lt;400&gt; 2

Asn	Asp	Asp	Cys	Glu	Leu	Cys	Val	Asn	Val	Ala	Cys	Thr	Gly	Cys	Leu
1					5				10					15	

&lt;210&gt; 3

&lt;211&gt; 15

&lt;212&gt; PRT

&lt;213&gt; Human guanylin

&lt;400&gt; 3

Pro	Gly	Thr	Cys	Glu	Ile	Cys	Ala	Tyr	Ala	Ala	Cys	Thr	Gly	Cys
-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

1 5 10 15

<210> 4  
<211> 19  
<212> PRT  
<213> Escherichia coli

<400> 4  
Asp Ser Ser Asn Tyr Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Thr  
1 5 10 15

Gly Cys Tyr

<210> 5  
<211> 19  
<212> PRT  
<213> Vibrio cholerae

<400> 5  
Leu Ile Ile Asp Cys Cys Glu Leu Cys Cys Asn Pro Ala Cys Phe Gly  
1 5 10 15

Cys Leu Asn

<210> 6  
<211> 18  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: PCR primer  
designed from regions flanking the open reading  
frame of human pre-prouroguanylin cDNA

<400> 6  
gaacccaggg agcgcgat 18

<210> 7  
<211> 19  
<212> DNA  
<213> Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: PCR primer  
designed from regions flanking the open reading  
frame of human pre-prouroguanylin cDNA

&lt;400&gt; 7

ctggtgggct cagggtacc

19

&lt;210&gt; 8

&lt;211&gt; 18

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Primer for  
RT-PCR or guanylin

&lt;400&gt; 8

aactcaggaa ctttgcac

18

&lt;210&gt; 9

&lt;211&gt; 18

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Primer for  
RT-PCR of guanylin

&lt;400&gt; 9

cgtaggcaca gatttcac

18

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/21998

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K7/08 A61K38/10 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, CHEM ABS Data, BIOSIS, WPI Data, PAJ, MEDLINE, EMBASE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	SHAILUBHAI, KUNWAR ET AL.: "UROGUANYLIN TREATMENT SUPPRESSES POLYP FORMATION IN THE APCMIN/+ MOUSE AND INDUCES APOPTOSIS IN HUMAN COLON ADENOCARCINOMA CELLS VIA CYCLIC GMP" CANCER RES (2000) 60(18) 5151-5157, XP002159386 the whole document	1-75
X	US 5 879 656 A (WALDMAN SCOTT A) 9 March 1999 (1999-03-09) abstract; claims 1-3, 50-55 column 8, line 40 - line 50 column 9, line 50 - line 60 -/--	1-75

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

Date of the actual completion of the international search

5 February 2001

Date of mailing of the international search report

21/02/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Cervigni, S

## INTERNATIONAL SEARCH REPORT

Int. :ional Application No

PCT/US 00/21998

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FORTE LEONARD R: "Guanylin regulatory peptides: Structures, biological activities mediated by cyclic GMP and pathobiology." REGULATORY PEPTIDES, vol. 81, no. 1-3, 31 May 1999 (1999-05-31), pages 25-39, XP000979549 ISSN: 0167-0115 page 36, paragraph 9.3 ----	1-75
X	WO 97 42220 A (UNIV JEFFERSON ;WALDMAN SCOTT A (US)) 13 November 1997 (1997-11-13) abstract page 12, last paragraph -page 13, paragraph 1 page 7, line 17 ----	2,53,63, 72,74
X	US 5 962 220 A (WALDMAN SCOTT A) 5 October 1999 (1999-10-05) abstract column 7 -column 8 ----	2,53,63, 72,74
X	WO 99 39748 A (MATTHEWS DEREK PETER ;NYCOMED IMAGING AS (NO)) 12 August 1999 (1999-08-12) abstract page 38 -page 39 ----	2,53,63, 72,74
A	FORSSMANN W -G ET AL: "REVIEW: GUANYLIN IS A NEW GASTROINTESTINAL HORMONE REGULATING WATER-ELECTROLYTE TRANSPORT IN THE GUT" FALK SYMPOSIUM,US,UNIVERSITY PARK PRESS, BALTIMORE, vol. 77, 12 June 1994 (1994-06-12), pages 279-292, XP000618033 ISSN: 0161-5580 -----	

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. l. Application No

PCT/US 00/21998

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5879656 A	09-03-1999	US 5518888 A AU 681920 B AU 8124994 A CA 2174928 A EP 0734264 A JP 9506340 T NO 961706 A WO 9511694 A US 6087109 A US 5962220 A US 6060037 A	21-05-1996 11-09-1997 22-05-1995 04-05-1995 02-10-1996 24-06-1997 20-06-1996 04-05-1995 11-07-2000 05-10-1999 09-05-2000
WO 9742220 A	13-11-1997	CA 2254082 A EP 0951475 A	13-11-1997 27-10-1999
US 5962220 A	05-10-1999	US 5518888 A US 6087109 A AU 681920 B AU 8124994 A CA 2174928 A EP 0734264 A JP 9506340 T NO 961706 A WO 9511694 A US 5879656 A US 6060037 A	21-05-1996 11-07-2000 11-09-1997 22-05-1995 04-05-1995 02-10-1996 24-06-1997 20-06-1996 04-05-1995 09-03-1999 09-05-2000
WO 9939748 A	12-08-1999	AU 2530199 A	23-08-1999

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☒ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**